J. Biochem. Mol. Biol. Vol. 28, No. 1 pp. 12~16 Januray 31, 1995

Determination of Branched-Chain α-Keto Acid Dehydrogenase Activity in Rat Tissues

Hyun Sook Kim* and Wayne A. Johnson[1]

Department of Food and Nutrition, College of Home Economics, Sookmyung Women's University, Seoul 140-742, Korea
[1]Department of Human Nutrition and Food Management The Ohio State University, Columbus, OH 43210, U.S.A.
(Received July 12, 1994)

Abstract: The branched-chain α-keto acid dehydrogenase (BCKAD) complex is a rate limiting enzyme which catalyzes the oxidative decarboxylation of branched-chain α-keto acids. Numerous studies have suggested that BCKAD is subject to covalent modification *in vitro* via phosphorylation and dephosphorylation, which are catalyzed by a specific kinase and phosphatase, respectively. The biggest difficulty in the assay of BCKAD activity is to arrest the interconversion between the active and inactive forms. BCKAD activity was determined from fresh rat heart and liver tissues using homogenizing and assay buffers containing inhibitors of phosphatase and kinase. The results suggest that a radiochemical assay using α-keto[1-^{14}C]-isovalerate as a substrate for the enzyme can be applied as a reliable method to determine *in vitro* enzyme activity with arrested interconversion between the active and inactive forms of the BCKAD complex.
Key words: BCKAD (Branched-chain α-keto Acid Dehydrogenase), enzyme activity, rat heart, rat liver, radiochemical assay.

Branched-chain amino acids share a common membrane-transport system and an enzyme responsible for their transamination; branched-chain amino acid transaminase-isoenzyme I (Block, 1989; Pelletier *et al.*, 1991). Subsequent irreversible oxidative decarboxylation is catalyzed by a mitochondrial branched-chain α-keto acid dehydrogenase (BCKAD) complex. The BCKAD complex has been postulated to be located on the outer side of the inner membrane of mitochondria (Johnson & Connelly, 1972a). Paxton and Harris (1982) purified BCKAD from rabbit liver and estimated the relative molecular weight at greater than 2×10^6 Da. The enzyme complex is similar to the pyruvate dehydrogenase complex (Wexler *et al.*, 1991), and is composed of three separate catalytic subunits held together by noncovalent interaction, which catalyze consecutive steps in the overall reaction: (a) branched-chain α-keto acid decarboxylase arranged in an α2β2 substructure with thiamine pyrophosphate as a prosthetic group (E1); (b) dihyrolipoyl transacylase with lipoate as a prosthetic group (E2); and (c) dihydrolipoyl dehydrogenase with flavin adenine dinucleotide as a prosthetic group (E3). Recently, investigators reported a deficiency of the E1 beta subunit (Hayashida *et al.*, 1994), and the E2 gene (Mitsubuchi *et al.*, 1991) in the BCKAD complex due to a single base substitution of intron 5. This resulted in two alternatively spliced mRNAs in a patient with maple syrup urine disease.

Further evidence is accumulating that BCKAD is covalently modified *in vitro* via phosphorylation and dephosphorylation (Odessey, 1980; Odessey, 1982; Harris *et al.*, 1982; Paxton and Harris, 1982), and it has been emphasized that comparisons of BCKAD activity under various physiological conditions, or in different tissues, must take into account its activation state. Thus, the biggest challenge in the assay of BCKAD activity is to arrest the interconversion between the active and inactive forms. In this report a radiochemical assay of BCKAD activity in rat heart and liver tissues is described.

Materials and Methods

Materials

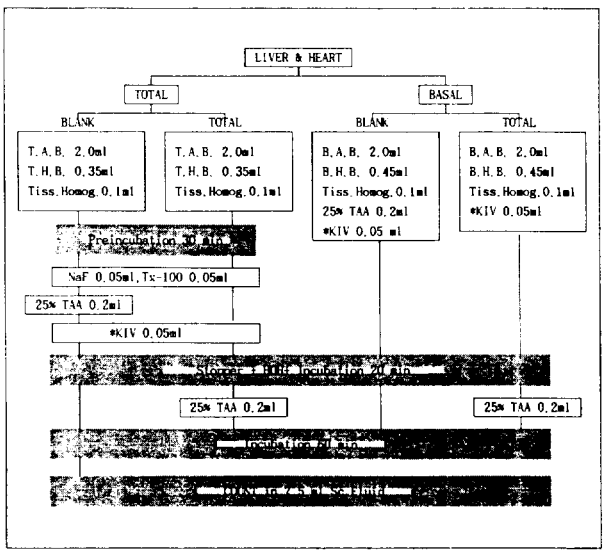
N,N,N′-tetraacetic acid, Tris-(hydroxymethyl) aminomethane, NaF, N-α-tosyl-l-lysine chloromethylketone, DL-dithiothreitol, trypsin inhibitor, cocarboxylase, coenzyme A, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid, and NAD^+ were purchased from Sigma Chemical Co. α-Chloroisocaproate was obtained from Drs. Robert J. Strohscheim and Ronald Simpson of Sandoz Inc., East Hanover, NJ, U.S.A. Rat liver and heart samples were obtained from male Sprague-Dawley rats

*To whom correspondence should be addressed.
Tel: (02) 710-9469, Fax: (02) 710-9470

Table 1. Components of homogenizing buffer

Hmogenizing buffer (pH 7.8)	
Ingredient	mM
Sucrose[a]	200
KCl[a]	50
$MgCl_2$[a]	5
α-chloroisocaproate[c]	0.1
Ethyleneglycol-bis(β-aminoethylether)-N,N,N'-tetraacetic acid[b]	5
Tris-(hydroxymethyl)aminomethane[b]	50
NaF[b]	50
N-α-tosyl-l-lysine chloromethylketone[b]	1
DL-dithiothreitol[b]	5
Triton X-100[d]	1 mg/ml
Trypsin inhibitor[b]	1 mg/ml

[a] Mallinckrodt, INC., Paris, KY.
[b] Sigma Chemical Co., St. Louis, MO.
[c] Gift from Drs. Strohscheim and Simpson of Sandoz Inc., East Hanover, NJ.
[d] U.S.Biochemical Corp., Cleveland, OH.



T. A. B. : Total assay buffer, T. H. B. : Total homogenizing buffer
B. A. B. : Basal assay buffer, B. H. B. : Basal homogenizing buffer
TAA : Trichloroacetic acid, HOH+ : Methylbenzothenium hydroxide
*KIV : ^{14}C-α-ketoisovalerate

Fig. 1. BCKAD assay procedure.

(Harlan Industries, Indianapolis, Indiana) weighing approximately 300 g.

Tissue preparation

The BCKAD complex is readily interconvertible between the active (dephosphorylated) and inactive (phosphorylated) forms. Therefore, it is crucial that a minimum amount of time should be spent in removing the tissues, and that all assays must be done at cold temperatures to minimize interconversion of the enzyme. The basal activity, which is the activity level determined when the *in vivo* phosphorylation state is preserved, was determined by an assay in which interconversion was arrested. Rats were euthanized by decapitation using a guillotine. The liver and heart were surgically removed (<30 sec) and kept ice cold. Only fresh tissues were used for enzyme activity measurement.

Enzyme assay

A radiochemical assay method using a liquid scintillation counter (Beckman Co., LS3801) was used, and the activity of BCKAD was determined by measuring the release of $^{14}CO_2$ from α-keto[1-^{14}C]-isovalerate (Amersham) using a modification of the method of Kasperek *et al.* (1989).

Homogenizing buffer was prepared as shown in Table 1. α-Isocaproate was used as a kinase inhibitor. NaF and N-α-tosyl-l-lysine chloromethyl-ketone are a phosphatase inhibitor and a protease inhibitor, respectively. Triton X-100 releases the dehydrogenase complex from mitochondria. KCl aids in extraction and stabilization of the enzyme (Goodwin *et al.*, 1988). α-Chloroisocaproate and ethyleneglycol-bis(β-aminoethylether)-N,N,N'-tetraacetic acid (EGTA) prevent enzyme inactivation by MgATP kinase. Protease inhibitor protects the enzyme against unusual sensitivity of the E2 subunit to proteolysis. The BCKAD complex is sensitive to agents which oxidize or bind SH groups, and to proteolysis. Therefore, it is necessary to include dithiothreitol in extraction and assay buffers (Patston *et al.*, 1988).

A summary flow chart of the enzyme assay procedures for basal and total activity is presented in Fig. 1. Approximately 0.3 g of fresh liver and heart tissues was quickly removed and homogenized in homogenizing buffer using a Potter-El Jehein glass homogenizer on ice. A 10% crude homogenate (i.e., 9 volumes of homogenizing buffer) was routinely prepared.

Assay buffer (Table 2) was prepared, and the assay itself was performed by incubating a portion of the crude homogenate (100 μl of liver and heart) made from fresh tissue in 2.0 ml of assay buffer at 37℃, pH 7.4. Homogenizing buffer was added to bring the volume to 2.8 ml. The reaction was started by adding 50 μl of α-keto[1-^{14}C]-isovalerate (4 mM, 0.01 μCi/50 μl). After incubating for 20 min, the reaction was stopped by adding 0.2 ml of 25% trichloroacetic acid. Liberated $^{14}CO_2$ was collected using methylbenzothenium hydroxide in a center well for 1 h, and counted in 7.5 ml of Scinti Verse E containing 10% water. Blanks were assayed in the same manner except the homogenate was poisoned with 0.2 ml of 25% trichloroacetic acid prior to addition of the keto acid. Total activity of BCKAD was determined after complete activation of the enzyme in homogenates made from fresh tissue.

Table 2. Components of assay buffer

Assay buffer (pH 7.4)	
Ingredient	mM
Mannitol[a]	200
Sucrose[b]	70
$MgCl_2$[b]	5
Cocarboxylase[a]	0.45
Coenzyme A[a]	0.8
NaF[a]	50
N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid[a]	2
NAD^+[a]	2
DL-dithiothreitol[a]	5
Triton X-100[c]	1 mg/ml

[a] Sigma Chemical Co., St. Louis, MO.
[b] Mallinckrodt, INC., Paris, KY.
[c] U.S.Biochemical Corp., Cleveland, OH.

The homogenizing and assay buffers for total activity were the same as the ones used for basal activity, except that NaF and Triton X-100 were omitted. Total BCKAD activity was assayed after first preincubating the tissue homogenate for 30 min in the assay buffer without NaF and Triton X-100. Homogenizing buffer was added to bring the volume to 2.8 ml. After 30 min of preincubation, the assay was initiated by adding NaF, Triton X-100, and then α-keto[1-^{14}C]-isovalerate. The activity of the BCKAD complex is expressed as nanomoles of α-ketoisovalerate oxidized per minute per gram of tissue.

Results and Discussion

The understanding of branched-chain amino and keto acid metabolism is essential, since they are used as therapeutic agents in various clinical situations including hepatic encephalopathy, muscle-protein wasting, trauma, uremia, sepsis, and premature infants (Adibi, 1980; Denne *et al.*, 1992; Manier *et al.*, 1992). The branched-chain α-keto acid dehydrogenase (BCKAD) complex is a rate limiting enzyme which catalyzes the oxidative decarboxylation of branched-chain keto acids. Investigators have reported various values for activity of the BCKAD complex since the assay technique for this enzyme is yet refined. The main difficulty in BCKAD activity assay is a reversible activation and inactivation cycle *in vitro* (Odessey, 1980; Odessey, 1982; Harris *et al.*, 1982; Paxton and Harris, 1982). The M_r= 46000 Da, α-subunit of the E1 component of the mitochondrial BCKAD complex is phosphorylated with MgATP and inactivated (Paxton and Harris, 1982; Odessey, 1980), but the molecular basis of enzyme phosphorylation is not fully understood. The activation

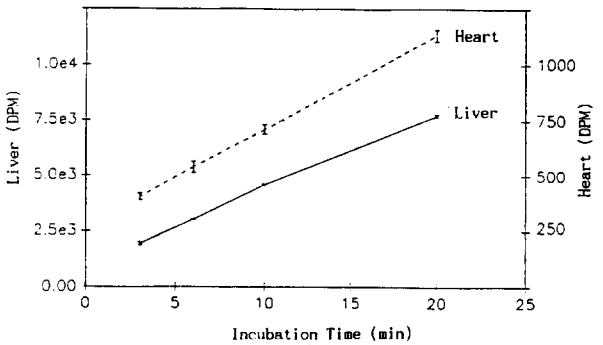


Fig. 2. $^{14}CO_2$ release at different incubation times.

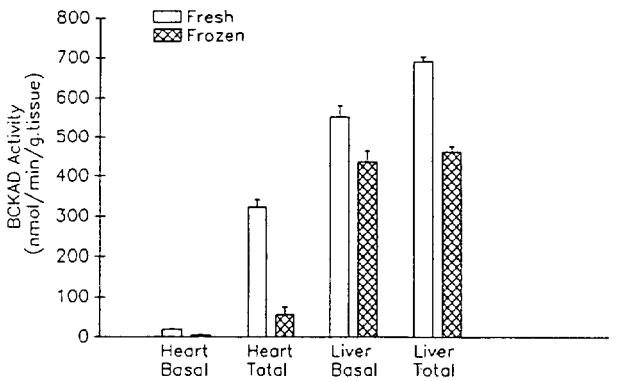


Fig. 3. Effect of freezing of tissue on BCKAD activity in rats.

of BCKAD is accomplished by a broad specificity protein phosphatase (Harris & Parker, 1982). Odessey (1980) reported that, in skeletal muscle mitochondria, the rate of inhibition by ATP is decreased by oxo acid substrates and enhanced by NADH, while BCKAD can be reactivated 10~20 fold by incubation at pH 7.8 in a buffer containing Mg^{2+} and cofactors. The assumption that no interconversion occurred during the assay was tested by performing the assay for varied periods of time using liver and heart homogenates in which BCKAD had been partially activated by a 10 min preincubation (Fig. 2). Linearity of the release of $^{14}CO_2$ with time was observed both in heart and liver homogenates, showing that the enzyme activity was unchanged during the assay. If BCKAD activity had increased or decreased during the assay, the plot would have curved up or down, respectively.

Other investigators have routinely used frozen tissues to determine BCKAD activity for convenience, assuming that freezing of tissue had no effect on the activity of the enzyme complex (Danner *et al.*, 1978; Sullivan *et al.*, 1976). To investigate whether freezing tissue has any impact on enzyme activity, one half of the removed hearts and livers were frozen in liquid nitrogen and assayed after three weeks. After freezing the basal and total activities were significantly decreased in both

Table 3. BCKAD activity and activity state in rat heart and liver

Tissue	BCKAD activity nmol/min/g tissue		Activity state (%)
	Basal	Total	
Heart	9.2± 3.5	317.3± 13.7	2.9± 1.9
Liver	604.8± 9.3	685.7± 18.1	88.5± 3.7

Values are Mean± SE for 12 rats in each group.

tissues (Fig. 3). Only 28.8± 1.1% and 17.8± 2.0% of basal and total enzyme activities were detected in frozen heart tissues. Basal and total activities of frozen liver tissue enzyme were also significantly decreased, compared to fresh tissue. These data support previous reports of a decrease in activity on freezing and thawing of tissues (May *et al.*, 1980; Odessey and Goldberg, 1979). Kasperek *et al.* (1985), using fresh tissue for the determination of total activity reported no change in basal activity in frozen tissue, which was not corroborated in this study. These results suggest that fresh tissues should always be used for the determination of BCKAD activity in homogenates and isolated mitochondrial preparations.

The branched-chain α-keto acids (α-ketoisocaproate, α-ketoisovalerate, and α-keto-β-methylvalerate) derived from transamination of corresponding amino acids (leucine, valine, and isoleucine), are the principal substrates used in the assay procedure, with half-maximum activity in the low micromolar range (Johnson and Connelly, 1972b). Activity is highest with α-ketoisovalerate, making this the substrate of choice for enzyme determination because of enhanced sensitivity (Goodwin *et al.*, 1988).

Enzyme activity and the activity state of BCKAD are shown in Table 3. Rat heart tissue had basal and total BCKAD activities of 9.2± 3.5 and 317.3± 13.7 nmol /min/g tissue, respectively, which were comparable to other reports (Wagenmakers *et al.*, 1984; Kasperek *et al.*, 1985). Liver tissue had a much higher enzyme activity under normal conditions. This phenomenon showed that nearly full enzyme activity is present under normal conditions, which has also been documented by other investigators (Gillim, 1983; Kasperek, 1985), but the mechanism has not yet been elucidated. Wagenmakers *et al.* (1985) reported basal and total liver BCKAD activities of 132± 13 and 136± 11 nmol/min/g tissue respectively, which are much lower than the results of this study. Total liver enzyme activity was reported as 364± 28 nmol/min/g tissue by Kasperek *et al.* (1985). Possible explanations of the discrepancies in reported values of enzyme activity could be: [1] use of different substrates for the enzyme complex, such as α-keto isocaproate, [2] freezing of tissue which affects enzyme activity, [3] variable contents of assay and homogenizing buffers, such as kinase, phosphatase, and protease inhibitors which may influence enzyme activity. The proportion of the BCKAD enzyme present in the active form in tissue is defined as the activity state of the enzyme, and extrapolated by determining the percentage of basal activity to total activity in the tissue. Only 2.9± 1.9% of the enzyme was present in the active form in heart tissue, while 88.5± 3.7% of the liver enzyme was in the active state. These values are comparable to other reports (Gillim *et al.*, 1983).

In conclusion, the enzyme activity of the branched-chain α-keto acid dehydrogenase complex in rat heart and liver tissues was determined by a radiochemical assay using α-keto[1-^{14}C]-isovalerate as a substrate for the enzyme. The results suggest that a reliable *in vitro* assessment of BCKAD activity can be performed with fresh tissues using proper homogenizing and assay buffers to arrest enzyme interconversion between phosphorylated and dephosphorylated forms during the assay.

References

Adibi, S. A. (1980) *J. Lab. Clin. Med.* **95**, 475.

Block, K. P. (1989) In: *Absorption and utilization of amino acids*. Vol. 1. pp. 229-244, CRC Press, Inc.

Danner, D. J., Lemmon, S. K. and Elsas, L. J. (1978) *Biochem. Med.* **19**, 27.

Denne, S. C., Kam, C. A. and Liechty, E. A. (1992) *Am. J. Clin. Nutr.* **56**, 899.

Gillim, S. E., Paxton, R., Cook, G. A. and Harris, R. A. (1983) *Biochem. Biophys. Res. Commun.* **111**, 74.

Goodwin, G. W., Zhang, B., Paxton, R. and Harris, R. A. (1988) In: *Methods in enzymology*, Vol. 166, Branched-chain amino acids (Harris, R. A. and Sokatch, J. R. ed) pp. 189-201, Academic Press INC.

Harris, R. A., Paxton, R. P. and DePaoli-Roach, A. A. (1982) *J. Biol. Chem.* **257**, 13915.

Hayashida, Y., Mitsubuchi, H., Indo, Y., Ohta, K., Endo, F., Wada, Y. and Matsuda, I. (1994) *Biochimica et Biophysica Acta* **1225**(3), 317.

Johnson, W. A. and Connelly, J. L. (1972a) *Biochemistry* **11**, 1967.

Johnson, W. A. and Connelly, J. L. (1972b) *Biochemistry* **11**, 2416.

Kasperek, G. J. (1989) *Am. J. Physiol.* **256**, E186.

Kasperek, G. J., Dohm, G. L. and Snider, R. D. (1985) *Am. J. Physiol.* **248**, R166.

May, M. E., Mancuci, V. J., Aftring, R. P. and Buse, M. G. (1980) *Am. J. Physiol.* **239**, E215.

Maniar, S., Laouari, D., Burtin, M, Parvy, P. R. and Kleinknecht, C. (1992) *Am. J. Clin. Nutr.* **56**, 1025.

Mitsubuchi, H., Nobukuni, Y., Akaboshi, I., Indo, Y., Endo, F. and Matsuda, I. (1991) *J. Clin. Invest.* **87**(4), 1207

Odessey, R. (1980) *Biochem. J.* **192**, 155.

Odessey, R. (1982) *Biochem. J.* **204**, 353.

Odessey, R. and Goldberg, A. L. (1979) *Biochem. J.* **178**, 475.

Patston, P. A., Espinal, J., Beggs, M. and Randle, P. J. (1988) In: *Methods in enzymology*, Vol. 166 Branched-chain amino acids (Harris, R. A. and Sokatch, J. R. ed) pp. 175-189, Academic Press INC.

Paxton, R. and Harris, R. (1982) *J. Biol. Chem.* **257**, 14433.

Pelletier, V., Marks, L., Wagner, D. A., Hoerr, R. A. and Young, V. R. (1991) *Am. J. Clin. Nutr.* **54**, 395.

Sullivan, S. G., Dancis, J. and Cox, R. P. (1976) *Arch. Biochem. Biophys.* **176**, 225.

Wagenmakers, A. J. M., Schepens, J. T. G. and Veerkamp, J. H. (1984) *Biochem. J. 223, 815.*

Wexler, I. D., Hemalatha, S. G. and Patel, M. S. (1991) FEBS Letters. **282**(1), 209.